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Cincinnati, Ohio

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ULTRAMICRO DETECTION OF BENZO(A)PYRENE IN THE AIR

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The rapid increase in death rates from lung cancer during the last twenty years has led to a search for possible environmental causes, of which tobacco smoking[33] and air pollutants[25] are the most suspected. Some evidence has been presented that the presence of benzo(a)pyrene in cigarette smoke and air may be involved in this increase[36].

In 1775 Pott[31] described scrotal cancer in chimney sweeps and traced it to contamination of the skin by chimney soot. Then in 1875 von Volkmann[43] reported occupational skin tumours among the workers in the tar and paraffin industry. In 1915 Yamagiwa and Ichikawa[46] artificially produced cancer by application of coal tar to the ears of rabbits. Later Tsutsui[40] showed the advantage of painting the skin of mice as a method of biological testing of possible carcinogenic materials. In 1922 strict proof of the carcinogenicity of soot was obtained when Passey[30] produced malignant growths by painting the skin of mice with an ethereal extract. More recently Kotin and his coworkers reported the production of skin cancers in mice following painting with extracts of Los Angeles atmosphere[26] and materials collected from gasoline[27] and diesel engine exhaust fumes[28].

In 1930 dibenzo(a,h)anthracene was found to have pronounced carcinogenic properties[24]. This was the first known pure chemical with potent cancer-causing activity. In 1933 a strongly carcinogenic crystalline fraction was separated from two tons of pitch. From this fraction a pure compound with potent cancer-causing activity was isolated[10,20] and subsequently shown through an unequivocal synthesis to be benzo(a)pyrene.[9] Since then hundreds of animal experiments with benzo(a)pyrene have

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repeatedly demonstrated its extreme potency as a cancer-causing agent[18].

On the basis of the similarity of the ultraviolet absorption spectra and/or the fluorescence spectra of appropriate chromatographic fractions of air particulate matter with the spectrum of benzo(a)pyrene, the atmosphere of several cities has been stated to contain benzo(a)-pyrene[11,12,17,26,36,44,45]. On the basis of similar evidence benzo(a)pyrene has been found in gasoline engine exhaust[27], diesel engine exhaust[28], and tobacco smoke[5,13,14].

From most of the estimates of the benzo(a)pyrene content in the air of various urban areas, it would appear that concentration is at a maximum in the winter and at a minimum during the summer. Larger cities appear to have a higher concentration than smaller cities or rural areas. In this respect Cooper[11] has reported the presence of 15-30 /100 m^3 of benzpyrene in the winter air of Salford, England. A comparison of the monthly benzpyrene content in the air in /100 m^3 has been made between Oslo, Norway and Bootle and Llangefni, Great Britain[4]. All communities had a peak in January or February. Llangefni and Oslo had values in the neighborhood of 1-2 while Bootle reached a peak of about 17 /100 m^3. For all cities the benzpyrene concentration dropped to a minimum in the summer months. A similar study has been reported for London air[44]. Here again a maximum concentration was reached in January with a value of about 15 /100 m^3. A minimum value of about 1 /100 m^3 was obtained in July. Although concentrations of other cities were lower, they also had similar variations in their atmospheric benzpyrene content. On the other hand, an analysis of Los Angeles air has shown the presence of approximately 3.3 of benzpyrene/100 m^3 during the so-called smog season

(August 1 to October 15, 1952) and 3 during the nonsmoggy season (October 21, 1952 to June 1, 1953)[26].

Identification of benzo(a)pyrene in the aromatic fractions from air, tobacco smokes, tars, etc., has been based on one or two of the following seven considerations[1-7]. We would like to add four additional identification methods which we have found to be of value in our air pollution investigations:

1. Melting point and mixed melting point with authentic benzo(a)-pyrene. This method of identification presupposes isolation of a visible amount of pure benzo(a)pyrene. Such an isolation from air particulate matter has apparently not yet been reported. As mentioned previously a slight amount of the compound has been isolated from two tons of coal tar pitch. Since then a relatively simple procedure has been developed whereby as much as 75 mg. of compound can be recovered from only 10 g. of a crude tar distillate[1]. Such a procedure might be applicable to the aromatic fraction of air particulate matter.

2. Ultraviolet absorption spectrum. In the past this method has proved to be the most useful for identification of benzo(a)pyrene. The reason can be seen by a glance at the ultraviolet spectrum of the compound (Figure 1). A fairly large number of sharp bands are present. The spectra of only three polynuclear hydrocarbons of the many reported show enough resemblances to the spectrum of benzo(a)pyrene to raise serious doubts of the identity of the compound (Table I). Of these three, only benzo[g,h,i]perylene is closely similar while the dibenzophenanthrene has not been detected in the air. However, detection of benzo(a)pyrene in the air has usually been based on the presence of bands at 366, 385,

TABLE I

Compound	λ_{max} (log ε)			
Benzo(a)pyrene[a]	225 (4.40)	255 (4.66) 266 (4.70) 284 (4.68) 297 (4.78)	330 (3.71) 347 (4.12) 366 (4.38) 385 (4.48)	403 (3.62)
Benzo[g,h,i]perylene[b]	>215	250 (4.2) 291 (4.7) 303 (4.8)	331 (3.8) 348 (4.0) 367 (4.4) 387 (4.5)	406 (2.75)
Dibenzo[b,g]phenanthrene[c]		248 (4.56) 284 (4.48) 295 (3.76) 308 (4.88)	338 (3.54) 354 (3.70) 372 (3.80) 391 (3.56)	401 (3.08)
9-Methylanthracene[d]		256 (5.2)	332 (3.4) 348 (3.7) 366 (3.9) 386 (3.9)	

[a] In alcohol, Reference 22.

[b] In benzene, Reference 6.

[c] In alcohol, Reference 7.

[d] In alcohol, Reference 23.

and 403 mμ, for at shorter wavelengths there is much more interference from difficultly separable contaminants. In Figure 2 the spectrum of such a fraction is shown. This particular fraction was obtained by chromatographic fractionation of the aromatic portion[37] of air particulate matter through alumina with pentane as the eluent. The ten ml. eluents obtained just before the dark yellow band came through showed definite peaks at 385 and 365 and inflections at 347 and 403 mμ. These eluents were combined, evaporated to near dryness, taken up in 20 ml. of pentane, and rechromatographed in a similar fashion. The fractionation procedure was essentially that described under the Internal standard method.

A procedure for differentiation between benzo(a)pyrene and benzo[g,h,i]perylene has been described by Cahnmann and Kuratsune[3]. The discrimination is based on a comparison of the near-visible spectra of these compounds in alcohol or benzene as compared to pentane. The spectrum of benzo(a)pyrene in pentane acquires two fine-structural bands at 375-380 mμ and another very weak band at approximately 393 mμ with the same order of intensity as the 403 mμ band. Benzoperylene does not show this effect. The previously described aromatic fraction showed this phenomenon.

After chromatographic fractionation and separation of fractions showing peaks at 365, 385, and 403 mμ, the benzpyrene content of the air fraction can be estimated by the Cooper procedure[12]. Linear background absorbance is assumed under the peak at 385 mμ. This peak is assumed to have a smaller contribution from contaminants than the much less intense peak at 403 mμ or the prominent peaks farther in the ultraviolet. A base line is drawn from a point on the absorption curve at 375 mμ to another

point on the curve at 395 mμ. A vertical line is dropped from the 385 peak to its intersection with the base line. The absorbance at the intersection of the base and vertical lines is then subtracted from the total absorbance at the 385 mμ peak. This is also done for a solution of known concentration of benzpyrene; then

$$\text{Concentration unknown} = \text{Concentration known} \times \frac{\text{Unknown absorbance}}{\text{Known absorbance}}$$

Cooper points out that the height of the benzpyrene peak at 403 mμ is about one-fifth of the height of its peak at 385 mμ. When the height of the 403 mμ peak is multiplied by 5.4, the value should give the height of the 385 mμ peak. If this is less than the height of the peak in the filtrate, Cooper believes the amount of benzperylene in this fraction can be estimated. Of course, this estimation depends on the absence of other contaminants absorbing near the peaks at 385 and 403 mμ. If the eluent containing benzo(a)pyrene is believed to have a fair amount of benzo[g,h,i]perylene, benzo(e)pyrene, or some other hydrocarbon, the variable reference technique described in detail by Jones, Clark, and Harrow[21] can be used. This spectrophotometric technique consists essentially of the gradual addition of a solution of the pure contaminant to the solvent in the reference cell until the contribution of the contaminant in the eluent is completely cancelled. The spectrum is determined after each small addition. By this method the presence of chrysene and benzo(a)anthracene has been determined in an eluate fraction from oysters[3].

Estimation of the amount of benzo(a)pyrene in the air has been based on the comparison of the ultraviolet or fluorescence spectrum of a standard benzo(a)pyrene solution to the ultraviolet or fluorescence spectra of chromatographic eluents which still contain a fairly large amount of

contaminants. These procedures are time-consuming and fairly complicated. The most that can be said for them is that they give a crude estimate of the amount of benzo(a)pyrene in the air. Urgently needed is a quick, simple, and fairly accurate method for this determination. With such a method, benzpyrene indices of local neighborhoods could be readily obtained. These could then be compared to the carcinogenic potency of the appropriate air fractions.

3. Fluorescence spectrum. Preliminary to the isolation of pure benzo(a)pyrene from tar, Mieger[19] investigated the fluorescence spectra of many carcinogenic mixtures, such as tars, mineral oils, etc. In all cases he found bands at 400, 418, and 440 mμ. This is of interest because aromatic fractions separated from the benzene-soluble fraction of air particulate matter[37] show fluorescence bands in acetone at 401 and 425 mμ with a shoulder at 455 mμ (Figure 3). On the other hand benzo(a)pyrene shows a much more intense spectrum with bands at 401, 425, and 455 mμ (Figure 3). Similarity of the fluorescence spectra does not, by itself, denote proof of structure. However, the method is highly sensitive. For example, the benzo(a)pyrene bands are detectable in a concentration of 1:20,000,000. It has been reported that benzo[g,h,i]perylene and 9-methylanthracene, which are somewhat similar spectrally in the near visible to benzo(a)pyrene, have very different fluorescence spectra from benzo(a)pyrene[5]. In acetone the fluorescent color of benzo(a)pyrene is blue while in trifluoroacetic-sulfuric acids a mixture of isomeric monocationic salts is formed which has a bright yellow fluorescence. Here we have another physical property that may prove valuable in analytical work. The fluorescent color of benzo[g,h,i]perylene is radically different

in trifluoroacetic-sulfuric acids, giving a bright blue color, while 9-methylanthracene gives a pink fluorescent color in the same solvent.

4. Internal standard method. To three equal portions of approximately 60 mg. of the aromatic fraction from air particulate matter was added 0, 1, and 2 ml. of 0.05% benzo(a)pyrene in pentane. Each mixture was separately chromatographed with pentane as an eluent on 2.5 g. of standardized[29] alumina in a 12-mm. wide column. Five ml. fractions were collected. Benzo(a)pyrene was eluted just before the dark yellow bands. Fluorescence was not used to follow the separation for it was found that the ultraviolet light tended to decompose the benzo(a)pyrene. In each fraction the pentane was evaporated at room temperature. The residue was dissolved in alcohol and the spectrum was obtained with a Cary Recording Spectrophotometer Model 11. Any fractions showing definite bands at 365, 385, and 403 mμ were analyzed at 385 mμ. In benzo(a)pyrene this band is the most intense in the near visible spectrum. Also this band shows the least interference from contaminant absorption at either shorter or longer wavelength. With this procedure the few aromatic fractions analyzed were found to contain from 1.0 to 1.6% of benzo(a)-pyrene type compounds. Under this designation we class benzo(a)pyrene and benzo[g,h,i]perylene.

5. Iodination of purified fractions. The aromatic fraction containing a fairly high concentration of the benzo(a)pyrene-like compound, as shown by the ultraviolet spectra, is iodinated. Any benzo(a)pyrene present is mostly converted to 6-iodobenzo(a)pyrene. This compound has peaks in its ultraviolet absorption spectrum at 402, 382 and 367 mμ with the 402 mμ band the most prominent. This method has been used for the

estimation of benzo(a)pyrene[41] and has also furnished additional proof for the presence of benzo(a)pyrene in tobacco smoke[5].

6. Diazotization method. Benzo(a)pyrene will combine with diazotized p-nitroaniline to give a brilliant purple dye, 6-(p-nitrobenzeneazo)benzo(a)pyrene[15,16]. With this color test benzo(a)pyrene can be detected in 1.2 parts per million. Benzo(a)pyrene has been reported in the Goose Barnacle (Mitella polymerus) on the basis of the spectral resemblance of a crystalline fraction treated with p-nitrobenzenediazonium chloride to pure 6-(p-nitrobenzeneazo)benzo(a)pyrene[32]. The difficulties in this test are that the color is slow in developing and only a few hydrocarbons have been tested.

7. Chromatography. Identification of benzpyrene is greatly aided by a knowledge of the relative position of adsorption on an adsorbent. For example, the order of adsorption of some hydrocarbons from cyclohexane solutions onto alumina are as follows: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, benzo(a)anthracene, chrysene, perylene, benzo(a)pyrene and benzo[g,h,i]perylene, anthanthrene, coronene[45]. A reproducible procedure for standardization of alumina for chromatographic separations of aromatic polycyclic hydrocarbons in gamma quantities has been described[29]. A comprehensive study of the chromatographic behavior of benzo(a)pyrene on partially deactivated silica gel columns has also been recently published[2]. Such standardization and simplification will be of great help in the air pollution field.

An aggressive research policy has been pursued by Falk and his coworkers who have developed ultramicro methods for the separation and identification of polynuclear aromatic hydrocarbons obtained from air

particulate matter[26]. Using activated alumina containing 2% added water, the air particulate extract was eluted with petroleum ether containing increasing concentrations of ether in steps of 2%. Benzpyrene was usually eluted by the 6% ether fraction. It was then identified by its chromatographic position, ultraviolet absorption and fluorescence spectra.

Paper chromatographic methods have been used for the separation and identification of benzpyrene. Tarbell et al.[8,38] have reported the R_f values of benzpyrene and other hydrocarbons in hexane saturated with dimethylformamide. In this system the R_f values and color under ultraviolet light were as follows: naphthacene, 0.51, orange; benzo(a)-pyrene, 0.54, blue; dibenzo[a,h]anthracene, 0.54, blue; pyrene, 0.62, green; benzo(a)anthracene, 0.66, blue; chrysene, 0.72, dark blue; fluoranthene, 0.82, green; and anthracene, 0.84, blue. Condensed polynuclear aromatic hydrocarbons are generally produced during the incomplete combustion of solid, liquid, or gaseous organic material[39]. Working with such combustion products, hydrocarbons such as anthracene, naphthacene, pyrene, fluoranthene, benzo(a)pyrene, perylene and dibenzo[a,h]-anthracene have been tentatively identified by paper chromatography mainly through the R_f value and fluorescent color. The following R_f values and fluorescent colors for benzo(a)pyrene and other hydrocarbons in 40% aqueous acetic acid as reported by these authors are as follows: dibenzo[a,h]anthracene, 0, yellow green; benzo(a)pyrene, 0.40, blue-violet; perylene, 0.47, gray yellow; benzo(a)anthracene, 0.54, violet blue; chrysene, 0.55, deep violet; fluoranthene, 0.71, gray white; pyrene, 0.71, blue; and anthracene, 0.90, violet. We have used 35% aqueous

dimethylformamide as a developing solvent in paper chromatographic separations of air pollutants. Benzo[g,h,i]perylene, benzo(a)pyrene, and 9-methylanthracene gave R_f values of 0.30, 0.42 and 1.0, respectively. This is another method for separating and/or identifying benzo(a)pyrene. The sensitivity of the piperonal chloride test with the spots on the chromatogram were of the order of 0.1 to 3 for the vast majority of polynuclear aromatic hydrocarbons. However, 0.001 of benzo(a)pyrene could be detected on filter paper by means of the blue fluorescence of this compound under ultraviolet light. Using the paper chromatographic procedure of Tarbell et al.[8,38], Van Duuren has identified benzo(a)pyrene and other hydrocarbons in tobacco smoke condensate on the basis of the R_f value, ultraviolet and fluorescence spectroscopy[42]. Many of these techniques, although applied to other fields, should be capable of application to the study of polluted air.

8. Nitration test. This test essentially involves nitration of aromatic material and then solution in alkaline dimethylformamide followed by a recording of the visible absorption spectrum. Of the many polynuclear hydrocarbons tested only pyrene and benzo(a)pyrene were found to give a blue color, max 620-630 mμ[34] (Figure 4). Since then benzo[g,h,i]perylene has been found to give the nitration test -- a blue color with bands at 375 and 620 mμ with the former band the strongest. For best results the nitrated product from benzpyrene or benzperylene had to be dissolved in dimethylformamide and then titrated with dilute tetraethylammonium hydroxide (1 ml. of 10% aqueous tetraethylammonium hydroxide diluted to 10 ml. with dimethylformamide) to the most intense blue color. Here again benzo(a)pyrene and benzo[g,h,i]perylene have

similar behavior. This test is actually superior for the detection of pyrene with which we are not concerned in this paper. By this test 15 of benzo(a)pyrene can be detected. The chromatographed air particulate fraction described in Method 2 (Figure 2) was tested by the nitration procedure. A purplish color was obtained with bands at 620 and 525 mμ. The 620 mμ band is apparently derived from benzo(a)pyrene or benzoperylene while the 525 mμ band is derived from contaminants.

9. Piperonal chloride test. Of all the tests for benzo(a)pyrene, this is probably the most sensitive. At least 1 part per million can be detected spectroscopically[35]. The dye that is formed has a yellow-brown color; the sensitivity of this test resides in the intense 750 mμ band which lies just outside the visible range of the eye (Figure 5). As piperonal chloride will react with almost any aromatic compound except benzene, quinones, and electronegatively substituted aromatic derivatives, the test for benzo(a)pyrene is not specific. The value of the test lies in the fact that the spectrum of the reaction mixture gives an idea as to its composition. This is because the simple aromatic compounds form orange dyes, and as the complexity of the aromatic compound increases, the color changes to red, violet, purple, blue, green, etc. The chromatographed air particulate fraction described in Method 2 (Figure 2) was tested by the piperonal chloride reaction. A green color was obtained with a max at 745 mμ and shoulders at 700 and 655 mμ. This would appear to indicate the presence of benzo(a)pyrene. However, benzo[g,h,i]perylene reacts with piperonal chloride to give a dye with an intense band at 760 mμ, while even 9-methylanthracene gives a band at 737 mμ. It is evident that the many claims in the literature for the isolation of

benzo(a)pyrene from air, cigarette smoke, tars, soots, etc. are based on the resemblance in the near visible spectrum of the fraction with the spectrum of pure benzo(a)pyrene. This evidence is equivocal, for benzo(a)pyrene and benzo[g,h,i]perylene in their ultraviolet spectra in alcohol (Table I) as well as their visible spectra obtained in both the nitration and piperonal chloride tests, show a remarkable similarity.

10. Protonation test. The resemblances between benzo(a)pyrene and benzo[g,h,i]perylene have been shown to be quite striking. Consequently a clear differentiation between these two hydrocarbons is a necessity. As previously indicated, their fluorescent spectra in acetone are entirely different. We have also found that the ultraviolet visible absorption spectra of these two compounds and of 9-methylanthracene in trifluoroacetic-sulfuric acids are quite different (Figure 6).

11. Aluminum chloride test. Anhydrous aluminum chloride is very soluble in nitromethane. As aluminum chloride is a very strong Lewis acid, use can be made of this property to form colored salts with polynuclear hydrocarbons which have the necessary basic properties. Quantitative results can thus be obtained. Benzo(a)pyrene gives a purple color in this test, the semiquantitative results of which are shown in Figure 7. Benzo[g,h,i]perylene and 9-methylanthracene give pink-violet and green colors, respectively, in the nitromethane-aluminum chloride test. The spectrum of each of these compounds in the solvent system is distinctly different (Figure 7).

CONCLUSION

Using the data presented here it is believed that an unequivocal identification of benzo(a)pyrene in air samples can now be made. With

the further evolution of these and newer, more nearly specific color tests for benzo(a)pyrene, development of a simple quantitative determination of benzo(a)pyrene in the air should follow.

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LIST OF FIGURES

Figure 1. Ultraviolet-visible absorption spectra of benzo(a)pyrene in 95% ethanol (-----) and in a trifluoroacetic acid solution containing 10% chloroform and 1% sulfuric acid (- - -).

Figure 2. Ultraviolet absorption in alcohol: Benzo(a)pyrene (-----) and an atmospheric chromatographic fraction (- - -).

Figure 3. Fluorescence spectra in acetone: Benzo(a)pyrene (-----) and the aromatic fraction from air particulate matter (- - -). Aminco Spectrophotofluorimeter was used at an activating wavelength of 378 mμ.

Figure 4. Visible absorption spectra: Nitration test on benzo(a)pyrene.

Figure 5. Ultraviolet-visible absorption spectra of benzo(a)pyrene: in alcohol (-----) and in a trifluoroacetic acid solution containing 17% chloroform and 0.035% piperonal chloride (- - -).

Figure 6. Ultraviolet-visible absorption spectra in trifluoroacetic acid containing 10% chloroform and 1% sulfuric acid: Benzo(a)pyrene (-----), benzo[g,h,i]perylene (- - -), and 9-methylanthracene (•••••), all 10^{-4} M.

Figure 7. Visible absorption spectra in nitromethane containing 20% aluminum chloride: Benzo(a)pyrene (-----), benzo[g,h,i]-perylene (- - -), and 9-methylanthracene (•••••), all 10^{-4} M.